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In situ regulation of DC subsets and T cells mediates tumor regression in mice

Omar A. Ali^{1,2,3}, Dwaine Emerich², Glenn Dranoff⁴, and David J. Mooney^{1,3}

¹ School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138

² InCytu Inc., 701 George Washington Hwy, Lincoln, RI 02865

³ Wyss Institute for Biologically Inspired Engineering, Cambridge, MA

⁴ Department of Medical Oncology and Cancer Vaccine Center, Dana-Farber Cancer Institute and Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

Abstract

Vaccines are largely ineffective for patients with established cancer, as advanced disease requires potent and sustained activation of CD8(+) cytotoxic T lymphocytes (CTL) to kill tumor cells and clear the disease. Recent studies have elucidated that subsets of dendritic cells (DCs) specialize in antigen cross-presentation and in the production of cytokines, which regulate both CTLs and T regulatory (Treg) cells that shut down effector T cell responses. In this study we have addressed the hypothesis that coordinated regulation of a DC network, and plasmacytoid DCs (pDCs) and CD8(+) DCs in particular, could dramatically enhance host immunity in mice. We used functionalized biomaterials incorporating various combinations of an inflammatory cytokine, immune danger signal and tumor lysates to control the activation and localization of host DC populations *in situ*. The numbers of pDCs and CD8(+) DCs, and the endogenous production of interleukin-12 (IL-12), all correlated strongly with the magnitude of protective anti-tumor immunity and the generation of potent CD8(+) CTLs. Vaccination by this method maintained local and systemic CTL responses for extended periods, while inhibiting FoxP3 Treg activity during antigen clearance, resulting in complete regression of distant and established melanoma tumors. The efficacy of this vaccine as a monotherapy against invasive and large tumors may be a result of the local activity of pDCs and CD8(+) DCs induced by persistent danger and antigen signaling at the vaccine site. These results indicate that a critical pattern of DC subsets correlates with the evolution of therapeutic, anti-tumor responses and provide a template for future vaccine design.

INTRODUCTION

Dendritic cells (DCs) are promising targets for immunotherapy as they play an essential role in initiating and regulating T cell immunity. The interaction between DCs and pathogens may eventually lead to antigen capture and processing by DCs. Pathogen associated molecular patterns (PAMPs), including Lipopolysaccharides (LPS) and cytosine-guanosine (CpG) rich sequences in pathogenic DNA, activate DCs via ligation of particular toll-like receptors (TLR),

Corresponding author: David J. Mooney, Harvard School of Engineering and Applied Sciences, 29 Oxford St., 319 Pierce Hall, Harvard University, Cambridge, MA 02138, Tel: 617-384-9624, mooneyd@seas.harvard.edu.

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Competing Interests: O.A., G.D., and D.J.M. have filed an application for a patent on the technology described in this manuscript. O.A. and D.E. are currently working for InCytu, Inc. to commercialize the technology.

which stimulate DC expression of specific costimulatory molecules and cytokines capable of propagating the appropriate T cell response. Activated DCs migrate to lymphoid tissues where they present pathogenic antigens and stimulatory molecules to naïve T cells, leading to T cell activation, expansion and specific responses.

Chronic exposure to tumor antigens with inappropriate co-stimulation and immunomodulation by T regulatory (T reg) cells allow solid tumors to develop by dysregulating DC activity and the cytotoxic T-lymphocyte (CTL) responses required to kill tumor cells^{1,2}. Cancer vaccines are frequently developed by using easily accessible, patient-derived blood monocytes that are transformed into DCs *ex vivo* with cytokine mixtures and pulsed with tumor antigens to promote antigen presentation³⁻⁵. These antigen-loaded DCs are then infused back into cancer patients with the goal of inducing anti-tumor immune responses mediated primarily by Th1 cells and CTLs³⁻⁵. Although clinical trials utilizing such *ex vivo* DC vaccines in advanced cancer patients have resulted in antigen-specific T-cell expansion and the production of protective cytokines *in vivo*²⁻⁶, many vaccines do not increase patients' survival over traditional treatments (for example, chemotherapy)² and have failed to consistently cause the regression of solid tumors¹⁻⁴. In both murine models and humans, these strategies are likely unable to generate the necessary numbers of functional CD8(+) CTLs, for the duration required to induce regression of solid, invasive tumors in both murine models and humans. Instead they may be amplifying defective CTLs that never become fully functional effectors at the tumor site, because of high local concentrations of immunosuppressive cytokines (for example, TGF- β and IL-10) and Tregs which dampen immune responses^{1,2}.

Hematopoietic precursor cells of both the myeloid and lymphoid lineage have the capacity to differentiate into two main categories of DCs, conventional DCs (cDCs) and plasmacytoid DCs (pDCs)⁷⁻⁹. Effective cancer vaccines may require both types of DCs, as each is equipped with a specific defense mechanism in response to invading pathogens. cDCs include CD11c+CD11b+ and CD11c+CD8+ cells, and exhibit classical DC morphology - protruding dendrites that make these cells especially adept at antigen processing and antigen presentation to T cells⁷⁻⁹. pDCs exhibit a spherical morphology⁷, and can produce large amounts of type-1 interferons (IFNs) in response to "danger signals", such as unmethylated cytosine-guanosine CpG dinucleotide sequences found in bacterial or viral DNA^{7,11,12}. pDC-derived type-1 IFNs link innate and adaptive immunity to viral infection by directly inducing naïve T-cell differentiation to Th1 cells¹¹⁻¹³ and by triggering antigen cross presentation to CD8+ T cells and interleukin production (for example, IL-12) by cDCs that facilitate the clonal expansion of CTLs. The plasticity of hematopoietic precursors likely allows for the recruitment and generation of the DC population most proficient at eliciting the appropriate immune response in a particular situation⁷⁻¹⁰. Current vaccines are unable to fully recapitulate *ex vivo* the development of this broad DC response, which is critical to the development of potent CTL immune responses^{3,4}.

Here we have hypothesized that one can manipulate the *in situ* generation of a heterogeneous DC network capable of CTL induction, and activate robust CD8(+) T cell effector responses to established tumors, by providing a secondary, immuno-stimulatory site of tumor antigen presentation. Inflammation or infection can produce DC populations that are not found in the steady state¹⁵, suggesting that stimuli in tissue microenvironments provoke a response from the network of DCs. The cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) is present at increased concentrations during inflammation^{16,17}, which may cause the recruitment of both monocytes and DCs while inducing local monocytes to differentiate into DCs^{15,17-19}. Recently, we described the development of implantable, synthetic polymer matrices that spatially and temporally control the *in vivo* presentation of cytokines, tumor antigens and danger signals¹⁴. GM-CSF was released from these poly(lactide-co-glycolide) (PLG) (an FDA approved biomaterial) matrices into the surrounding tissue, to recruit DC

precursors and DCs. CpG-rich oligonucleotides were immobilized on the matrices as danger signals, and antigen (tumor lysates) was released to matrix resident DCs to program DC development and maturation. These matrices quantitatively regulated DC activation and trafficking *in situ*, and induced prophylactic immunity against inoculations of murine, B16-F10 melanoma cells²⁶. Here, the ability of this system to control the recruitment and activation of multiple DC and T cell subsets and to provide therapeutic vaccination against established tumors was investigated.

RESULTS

Local GM-CSF delivery promotes recruitment of CD11b(+) DCs

As described previously¹⁴, macroporous PLG matrices were fabricated for GM-CSF release to recruit DCs, and with an interconnected porous structure that allows for cell infiltration.¹¹ Matrices were loaded with 0, 3000, and 7000 ng of GM-CSF and implanted into the subcutaneous pockets of C57BL/6J mice. Histological analysis at day 14 post-implantation of PLG matrices loaded with 3000 ng of GM-CSF revealed enhanced cellular infiltration when compared to blank controls (Fig. 1A). FACS analysis for CD11c(+) DCs showed that GM-CSF delivery recruited significantly more DCs (~8 fold increase) than blank PLG matrices (Fig. 1B). The matrix-resident DCs were almost exclusively CD11b+ (~87%), in accordance with other reports of GM-CSF effects on DC recruitment *in vivo*^{21,22}. The total number of DCs recruited and their expression of the co-stimulatory molecule CD86 increased with GM-CSF delivery in a dose dependent manner (Fig. 1D). However, the highest dose (7000 ng) of GM-CSF reduced the number of activated DCs at the implant site, as indicated by diminished MHCII and CCR7 expression at day 14 after implantation (Fig 1D & S1). Since total DC recruitment and activation both peaked at 3000ng GM-CSF, this dose was utilized to recruit and generate DCs in subsequent studies. GM-CSF delivery promoted greater cellular penetration into and association with the PLG material, as indicated by histological analysis (Fig. S1) and the increase in DC numbers (Fig. 1B & 1D), potentially allowing for the subsequent programming of resident DC precursors and DCs.

In situ delivery of CpG-ODN promotes pDC recruitment and IFN production

The ability of local presentation of danger signals to regulate the ratio of distinct DC subtypes was next examined, by immobilizing TLR-activating, polyethylenimine (PEI)-condensed CpG-ODN molecules, into the matrices. As described previously for plasmid DNA¹⁴, condensation of oligonucleotides with the polycationic polymer PEI results in positively charged particles that bind electrostatically to the anionic PLG matrix. PLG matrices incorporating CpG-ODN alone recruited CD11c(+)PDCA-1(+) pDCs to the PLG matrix(Fig. 2A), and this effect was enhanced with co-administration of GM-CSF (Fig. 2B). We altered the dose of CpG-ODN presented in combination with 3000ng GM-CSF to regulate the numbers of resident pDCs, resulting in 190,000, 520,000, and 1,200,000 cells at doses of 0, 10 and 100 µg of CpG-ODN, respectively (Fig. 2B). Co-presentation of CpG-ODN had little effect on the ability of GM-CSF to enhance CD11c+CD11b+ cDCs (Fig. 2C). High doses of CpG-ODN promoted the local production of IFN-α (~1010 pg/ml) and IFN-γ (~600 pg/ml), independently of the presence of GM-CSF (Fig. 2E & 2F). These results indicate that controlled GM-CSF and CpG-ODN danger signaling from synthetic extra-cellular matrices cooperate to regulate resident pDC and CD11c(+)CD11b(+) cDC numbers, along with the production of protective cytokines commonly linked to Th1 and CTL immunity.

Tumor lysate co-delivery with CpG-ODN and GM-CSF stimulates CD8(+) generation and IL-12 production

We then hypothesized that co-presenting cancer antigens with CpG-ODNs to matrix-resident DCs would promote further DC development, activation and CTL antigen sensitization. In this

context, necrotic tumor cells may be particularly immunostimulatory, as they release a variety of endogenous mediators (for example, heat shock proteins and damaged nucleic acids) that trigger innate immune recognition²⁴. Thus, freeze-thaw lysates of B16 melanomas were prepared, and antigen-presenting matrices were fabricated by encapsulating these lysates into the PLG material, resulting in localized and sustained antigen presentation to the infiltrating cell population¹⁴. These antigen-presenting matrices unexpectedly stimulated CD8(+) DC generation *in situ* (Fig. 3A). Upon viral invasion, CD8+CD11c+ cDCs are especially efficient at cross presenting exogenous antigen on MHC-I molecules^{10,25–28}, and at producing the T helper (Th)-1 promoting cytokine IL-12^{10,29–32}, which are two mechanisms that aid in priming cytotoxic T lymphocyte (CTL) immunity to viruses and tumors. This activity, however, is normally associated with lymphoid tissues^{7,9,25–28}. Co-presentation of tumor lysates with CpG-ODN led to the presence of 200,000 CD8(+) DCs, which increased to approximately 670,000 (9-fold increase over blank matrices) when GM-CSF was added to stimulate recruitment (Fig. 3B). Additionally, tumor lysate in combination with GM-CSF and CpG enhanced the numbers of recruited pDCs at Day 10 after implantation by two-fold over matrices without lysate, and by 10-fold over blank controls (Fig 3C). No significant difference in pDC numbers was observed with tumor lysate in combination with only GM-CSF or CpG signaling. The CD11c(+)CD11b(+) DC population at the vaccine site depended on GM-CSF alone (Fig 3D), as tumor lysate or CpG signaling alone or in combination had no significant effect on the recruitment and expansion of these DCs (Fig 3D).

It is interesting that the *in situ* production of the T-cell growth factor IL-12 at matrices that deliver both tumor lysate and CpG-ODN to cell populations recruited by GM-CSF was approximately four-fold higher than at blank matrices, and at least two-fold higher than at all other matrix formulations (Fig 3E). However, tumor lysates in the matrix did not increase the high levels of IFN- α and IFN- γ induced by CpG-ODN alone (Fig 3F & 3G). These results suggest that the engineered matrices manipulated both the number and function of specific DC subsets, as well as the accompanying CTL-polarizing activity.

PLG matrices co-delivering GM-CSF, CpG-ODN, and tumor lysates stimulate potent local and systemic CD8(+) cytotoxic T cells

To elucidate the adaptive immune mechanisms induced by PLG vaccines that deliver tumor lysate, GM-CSF and CpG-ODN, the activity of both local and systemic CTLs were examined. Flow cytometric analysis of cells infiltrating the vaccine site revealed a significant CD3(+) CD8(+) T cell response by day 5 (representative sample $\sim 1.9 \times 10^5$ cells), which peaked at Day 12 when a relatively large proportion of the matrix-resident cells were CTLs (representative sample: 8.5% of cells; $\sim 8.5 \times 10^5$ cells) (Fig. 4A). Local CD8(+) T cell numbers dropped sharply by day 16 and were negligible at day 21 (Fig. 4B), likely due to antigen clearance. PLG vaccines containing tumor lysates, GM-CSF and CpG-ODN preferentially tuned and promoted CD8(+) cytotoxic immune responses, relative to other matrix formulations devoid of CpG (Fig 4C). Further, the activation and persistence of systemic CTL responses was monitored by staining splenocytes with MHC class I/TRP2 peptide pentamers to identify CTLs with specificity to tyrosinase-related protein (TRP)-2, which is a major antigenic target of melanoma vaccines in mice and humans. A significant expansion of TRP2-specific CTLs was observed in the spleens of vaccinated mice by day 5, which continued and peaked between days 7 and 16, before falling at days 21 through 28 (Fig. 4D & 4E), indicating that systemic anti-melanoma responses were being generated and sustained for extended periods.

Tumor protection induced by PLG matrices correlated to DC subsets and IL-12 production

It was previously demonstrated that this system is capable of generating prophylactic immunity against poorly immunogenic, B16-F10 melanoma¹⁴, and the relation of this anti-tumor efficacy to the specific DC networks invoked by various vaccine formulations was investigated. C57BL/

6J mice were vaccinated with PLG-based matrices incorporating B16 tumor lysates, GM-CSF, and CpG-ODN in varying combinations, and then challenged with live B16-F10 melanoma tumor cells at D14 after vaccination. PLG vaccines with both B16-F10 tumor lysates and either 1, 10, 50 or 100 µg doses of CpG-ODN danger signaling allowed 10–30% of the vaccinated mice to survive, tumor-free (Fig 5A), after an otherwise lethal cell challenge, while 100% of unvaccinated mice were euthanized by day 23 due to tumor burden. When GM-CSF mediated DC recruitment was combined with lysate and CpG-ODN delivery, the mice showed significant protection from tumor-induced lethality. CpG-ODN doses of 10, 50, and 100 µg resulted in 50, 60 and 90% survival rates (Fig. 5B).

The ability of vaccine systems to create a heterogeneous DC population correlated with the marked increase in anti-tumor efficacy. In comparison to antigen-matrices delivering GM-CSF alone, the antigen loaded-matrices delivering CpG and GM-CSF together resulted in a higher proportion of pDCs (~31% vs. 7%) and CD8+ cDCs (~14% vs. 5.5%) (Fig 5C), which correlated with a significant enhancement in mouse survival (90% vs. 20%), even though total DC numbers *in situ* were statistically similar (3.0 ± 0.6 vs. 4.2 ± 0.9 million DCs; two-tailed student's T test, $n=5$). Survival rates were proportional to the number of pDCs and CD8(+) cDCs, but not CD11b(+) DCs, generated at the PLG vaccine site at day 10 (Fig. 5D-5F). Additionally, the endogenous production of IL-12 was correlated with animal survival (Fig. 5G) suggesting the importance of cross-presentation and Th1 promoting cytokines to vaccine efficacy.

Engineered PLG matrices incorporating CpG-ODN attenuate immune regulation by FoxP3(+) Treg number and immunosuppressive cytokines

Although several vaccines designed to program DCs either *ex vivo* or *in situ* have achieved significant and long-term prophylactic protection in mouse models of cancer^{1,4,15}, eradication of invasive and well-established tumors has not been achieved without adoptive T cell transfer or systemic therapies^{1,2,33,34}. This limitation might reflect, at least in part, the ability of dendritic cell-based vaccines to stimulate Tregs^{35,36} that attenuate the cytotoxic activity of adaptive immune responses. Thus, we characterized the impact of the engineered matrices on the induction of immunosuppressive pathways. Monitoring CD4+ T cell responses to antigen-presenting matrices with GM-CSF and CpG revealed peak activity at days 5 and 7, which decreased to negligible levels by day 12 post-implantation (Fig 6A). In contrast, matrices containing GM-CSF and tumor lysate led to a significant enhancement of CD4(+) T cell infiltration at day 12 (Fig. 6B), and these cells likely contribute to regulation of CTL responses. Incorporation of GM-CSF and tumor lysate into the vaccine matrix led to a ten-fold increase in TGF-β levels (Fig. 6C), and a significant increase in IL-10 (Fig. 6D) at the vaccine site; these are cytokines commonly associated with Treg activity and immunosuppression. Further, as observed previously in GM-CSF based vaccines^{35,36}, GM-CSF co-signaling with tumor antigens resulted in a significant CD3(+)FoxP3(+) response at the vaccine site (Fig. 6E & 6F) when compared to all other matrix formulations, resulting in an almost even ratio of CD8(+) effectors and FoxP3 Tregs (Fig 6G). CpG-ODN presentation in concert with both tumor lysate and GM-CSF counteracted these immunosuppressive mechanisms, as TGF-β and IL-10 levels and Treg activity were not enhanced over the control matrices, and CD8(+) CTLs outnumbered FoxP3(+) T cells by approximately 25-fold at day 12 after implantation (Fig. 6C-G). Altogether, these findings suggest that this vaccine system is able to promote and extend CTL responses, likely through naïve T cell differentiation induced by pDCs and CD8(+) DCs, the corresponding production of type I-IFNs and IL-12, and inhibition of negative feedback mechanisms.

Engineered PLG matrices cause regression of established melanoma

Because a high ratio of CD8⁺ T cell effectors to FoxP3⁺ Tregs has been linked to therapeutic tumor immunity in murine and human systems^{35, 37}, we tested the activity of PLG vaccines against B16-F10 tumors that had been established for 9 days (inoculation of 5×10^5 cells at day 0). Tumor-bearing mice implanted with blank PLG matrices displayed rapid tumor growth and were euthanized by day 24, as expected (Fig. 7A). Vaccination of mice once with the PLG vaccine significantly decreased the rate of tumor progression (Fig. 7A), and an increase in mean survival time over controls was observed, but all animals required euthanization by day 58, depending on the tumor size at the time of vaccination (Fig. 7B). Vaccination of mice twice (days 9 and 19) with PLG vaccines had a more dramatic effect on tumor progression, and caused complete regression of tumors in a subset (7 of 15) of the animals (Fig. 7C). In contrast, a single treatment with irradiated, GM-CSF secreting B16-F10 cells, a widely used cell therapy currently in clinical trials, attenuated tumor progression modestly, and all animals had to be euthanized by day 36 (Fig. 7A). Tumor antigen presentation from PLG matrices enhanced protection and was required to induce tumor regression, as matrices with GM-CSF and CpG did not enhance survival times significantly (Fig 7A & B). Strikingly, 47% of the mice (animals bearing day 9 tumors) vaccinated twice with PLG vaccines survived long-term and free of detectable tumors; this treatment regimen was able to completely eradicate tumors of up to 25mm² in size (Fig. 7C).

To test whether PLG vaccines could be effective against an even greater tumor burden, melanoma tumors were established for 13 days, and the mice were then vaccinated. One-time (day 13) and two-time (day 13 and 23) vaccination decreased tumor progression (Fig. 7D). Two-time vaccination doubled the mean survival time, and led to complete tumor regression in 20% of the animals with advanced, solid tumors (day 13 tumors; n=15) (Fig. 7D-F).

Since vaccinations were initiated at days 9 and 13 of tumor growth, and required 5 days for CTL generation, the effector window for immune responses was small (~6–10 days) before untreated animals succumbed to tumor burden. Variations in tumor size at the time of vaccination likely accounted for the lack of complete regression in all animals; vaccination may not have resulted in generation of sufficient numbers of killer T cell in time to control and clear larger tumors. Slight hair loss and depigmentation was observed at the vaccine site, in agreement with the past study using this vaccination system¹⁴, but no significant toxicities were observed with vaccination.

DISCUSSION

Current approaches to cancer vaccination augment cellular and humoral anti-tumor reactions in many patients, but most immunized subjects still succumb to progressive disease, indicating that vaccine responses are insufficient to effectuate complete tumor cell killing^{1–4,38}. Nonetheless, the abilities of blocking antibodies to cytotoxic T lymphocyte associated antigen-4 (CTLA-4) and the adoptive transfer of T cells in lymphodepleted hosts to accomplish tumor regressions highlight the potential for immune-mediated destruction of advanced cancer^{37,39}. In this study, we demonstrate that the appropriate regulation of the DC network can induce complete regressions of distant and established melanomas in mice.

Our engineered PLG vaccine evokes a coordinated response of multiple DC subtypes, which together trigger sustained and potent anti-tumor CD8⁺ CTLs while inhibiting immunoregulatory pathways. The combination of tumor cell lysates, GM-CSF, and CpG-ODN in the vaccine matrix were required for optimal tumor protection, which was strongly associated with the recruitment of pDCs and CD8⁺ DCs and the local production of IL-12. The accumulation of CD8⁺ DCs at the vaccine site is a notable feature of this vaccination strategy, since this DC subset is typically localized to secondary lymphoid structures. Plasmacytoid DC

numbers were closely linked with the generation of type I-IFNs, as described in previous reports^{21,11,40}, and these cells helped to support the activation of CD8⁺ DCs and their cross-presentation of tumor antigens to Th1 cells and CTLs^{7–10,29}. Further studies to elucidate which of the correlative observations in this study underlie the superior anti-tumor effect are warranted, along with further analysis of the mechanisms by which the vaccine may modulate other components of the immune system (for example, myeloid derived suppressor cells that suppress GM-CSF based vaccines⁴¹). However, many cancer vaccination strategies use GM-CSF-stimulated monocyte-derived cDCs^{3–5} as immunogens, and our experiments do indicate that the presence of a broader set of DC subtypes may evoke more potent anti-tumor responses.

Our findings also suggest that a minimum number of DCs may be required to induce high levels of protective immunity. Vaccines that generated approximately 1,200,000 pDCs and 600,000 CD8⁺ DCs (~43% of total DCs) in a total population of approximately 4.2 million DCs resulted in 90% survival in a subsequent tumor challenge. The engineered matrices appear to program T cell responses efficiently by providing a site of sustained, immunostimulatory tumor antigen presentation, which evokes robust CTLs, both locally and systemically, and attenuates immune regulation mediated through TGF- β , IL-10, and FoxP3⁺ Tregs. The kinetics of the adaptive immune response to our system suggest that CTLs manifested potent effector function, as vaccination resulted in a prototypical activation phase that gradually plateaued, followed by a contraction phase as antigen was cleared. Other vaccine formulations achieve only short-lived stimulation with infusions of protein or manipulated cells and may not trigger this T effector profile, but instead induce at least partially dysfunctional T cells that are more likely to undergo exhaustion within the immunosuppressive tumor microenvironment^{1–4}.

Taken together, our results highlight a critical array of DC subtypes that are generated during the evolution of therapeutic anti-tumor responses in mice, which may provide a template for rational vaccine design more generally. Indeed, the vaccine system reported here might be adapted to modulate DC and CTL responses for the control of other solid cancers and perhaps chronic infections. Our approach might also facilitate the study of DC subset development and the mechanisms through which these subsets are coordinated *in vivo* for the eradication of established diseases. It is striking that tumor regression induced by these PLG vaccines outperformed gene-modified tumor-cell vaccines (GMTV) in direct comparison and outperformed *ex vivo* DC vaccines reported in literature^{1,2,4}. This acellular biomaterial system was designed with components that are either FDA approved (PLG and GM-CSF) or have been utilized clinically (CPG-ODN), and does not require the maintenance and modification of live cell cultures. Together, these features suggest this PLG system may have considerable advantages in terms of clinical application as compared to other approaches reported to date. Scaling to humans will likely not require significant modification of the size or structure of the material, but will require utilizing effective human analogs (for example, human GM-CSF and CPG-ODN sequences) that mediate similar DC and CTL responses. It is not clear whether the current combination of GM-CSF, CpG-ODN and tumor antigen is the optimal formulation, and other dosing regimens or alternative TLR agonists or cytokines should be tested.

METHODS

Matrix Fabrication

A 85:15, 120 kD copolymer of D, L-lactide and glycolide (PLG) (Alkermes) was utilized in a gas-foaming process to form porous PLG matrices²⁰. In brief, PLG microspheres encapsulating GM-CSF were first made by using standard double emulsion⁴². PLG microspheres were then mixed with 150 mg of the porogen, sucrose (sieved to a particle size between 250 μ m and 425 μ m), and compression molded. The resulting disc was allowed to equilibrate within a high-pressure CO₂ environment, and a rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure²⁰. The sucrose was leached from the scaffolds

by immersion in water yielding scaffolds that were 90% porous. To incorporate tumor lysates into PLG scaffolds, biopsies of B16-F10 tumors that had grown subcutaneously in the backs of C57BL/6J mice (Jackson Laboratory), were digested in collagenase (250 U/ml) (Worthington) and suspended at a concentration equivalent to 10^7 cells per ml after filtration through 40 μ m cell strainers. The tumor cell suspension was subjected to 4 cycles of rapid freeze in liquid nitrogen and thaw (37°C) and then centrifuged at 400 rpm for 10 min. The supernatant (1ml) containing tumor lysates was collected, incubated with the PLG microspheres and lyophilized and the resulting mixture was utilized in the high-pressure, CO_2 process to foam macroporous, PLG matrices incorporating tumor lysates. To incorporate CpG-ODNs into PLG scaffolds, CpG-ODN 1826, 5'-tcc atg acg ttc ctg acg tt-3', (Invivogen) was first condensed with poly(ethylenimine) (PEI, $M_n \sim 60,000$) molecules by dropping ODN-1826 solutions into PEI solution, while vortexing the mixture^{20,23}. The charge ratio between PEI and CpG-ODN ($\text{NH}_3^+:\text{PO}_4^-$) was kept constant at 7 during condensation. PEI-CpG-ODN condensate solutions were then vortexed with 60 μ l of 50% (wt/vol) sucrose solution, lyophilized and mixed with dry sucrose to a final weight of 150 mg. The sucrose containing PEI-CpG-ODN condensate was then mixed with blank, GM-CSF and/or tumor lysate loaded PLG microspheres to make PLG cancer vaccines.

In situ identification of DC subsets and T cells

Blank PLG matrices and matrices containing 3000ng GM-CSF alone or in combination with either 1, 10, 50, or 100 μ g CpG-ODN (studies were also performed with tumor lysates co-presented with either 3000ng GM-CSF or 100 μ g CpG-ODN alone or in combination) were implanted into subcutaneous pockets on the back of 7–9 week old male C57BL/6J mice. For histological examination, scaffolds were excised and fixed in Z-fix solution (Anatech Ltd), embedded in paraffin, and stained with hematoxylin and eosin. To analyze DC recruitment, scaffolds were excised at various time-points and the ingrown tissue was digested into single cell suspensions using a collagenase solution (Worthington, 250 U/ml) that was agitated at 37°C for 45 minutes. The cell suspensions were then poured through a 40 μ m cell strainer to isolate cells from scaffold particles and the cells were pelleted and washed with cold PBS and counted using a Z2 coulter counter (Beckman Coulter). To assess DC infiltration and activation, subsets of the total cell population isolated from PLG matrices were then stained with primary antibodies (BD Pharmingen) conjugated to fluorescent markers to allow for analysis by flow cytometry. Allophycocyanin (APC)-conjugated CD11c (dendritic cell marker), and Phycoerythrin PE-conjugated CD86 (B7, costimulatory molecule) stains were conducted for DC recruitment analysis, and APC-conjugated CD11c, fluorescein isothiocyanate (FITC)-conjugated CCR7, and PE-conjugated MHCII stains were conducted for DC programming analysis. To further delineate the presence of specific DC subsets, cells were also stained with APC-conjugated CD11c and PE-conjugated PDCA-1 (plasmacytoid DC marker) or APC-conjugated CD11c and PE-conjugated CD8 (CD8 DCs) or APC-conjugated CD11c and FITC-conjugated CD11b (CD11b DCs). To assess T cell infiltration, PE-Cy7 conjugated CD3 stains were performed in conjunction with APC-conjugated CD8a (CD8 T cells), FITC-conjugated CD4 (CD4 T cells), and PE-conjugated FoxP3 (Treg) and analyzed with flow cytometry. Cells were gated according to positive FITC, APC and PE with isotype controls, and the percentage of cells staining positive for each surface antigen was recorded.

Tumor growth assays, protective cytokines and Trp2 pentamer analysis

PLG scaffolds with melanoma tumor lysates and various dosages of GM-CSF and/or various quantities of PEI-CpG-ODN condensates were implanted subcutaneously into the lower left flank of C57BL/6J mice. For prophylactic vaccinations, animals were challenged 14 days later with a subcutaneous injection of 10^5 B16-F10 melanoma cells (ATCC) in the back of the neck. Animals were monitored for the onset of tumor growth (approximately 1mm^3) and sacrificed for humane reasons when tumors grew to 20 – 25 mm (longest diameter).

To assess PLG vaccine efficacy in the therapeutic setting, C57/BL6J mice were challenged with a subcutaneous injection of 5×10^5 B16-F10 melanoma cells (ATCC) in the back of the neck. At either days 9 or 13 after tumor challenge, PLG vaccines loaded with 3000ng GM-CSF, 100 μ g CpG-ODN and tumor lysates were implanted subcutaneously into the lower left flank of C57BL/6J mice. A subset of mice were vaccinated again at 10 days after the initial vaccination (days 19 and 23).

To determine in vivo IL-12p70, IFN- α , IFN- γ , and TGF- β concentrations at the matrix implant site, adjacent tissue was excised and digested with tissue protein extraction reagent (Pierce). After centrifugation, the concentrations of IL-12, IFN- α , IFN- γ , and TGF- β in the supernatant were then analyzed with ELISA (R&D Systems), according to the manufacturers instructions.

To determine the generation of TRP-2-specific cytotoxic T lymphocytes, single cell suspensions were prepared from the spleens of mice immunized with PLG vaccines (Lysate + 3000ng GM-CSF + 100 μ g CpG) at various timepoints. These cells were initially stained with APC-H-2Kb/TRP2 pentamers (Proimmune), and subsequently stained with PE-anti-CD8 mAb (mAb (BD Pharmingen) before being analyzed by flow cytometry.

Statistical analysis—All values in the present study were expressed as mean \pm S.D. The significant differences between the groups were analyzed by a Student's *t* test and a *P* value of less than 0.05 was considered significant.

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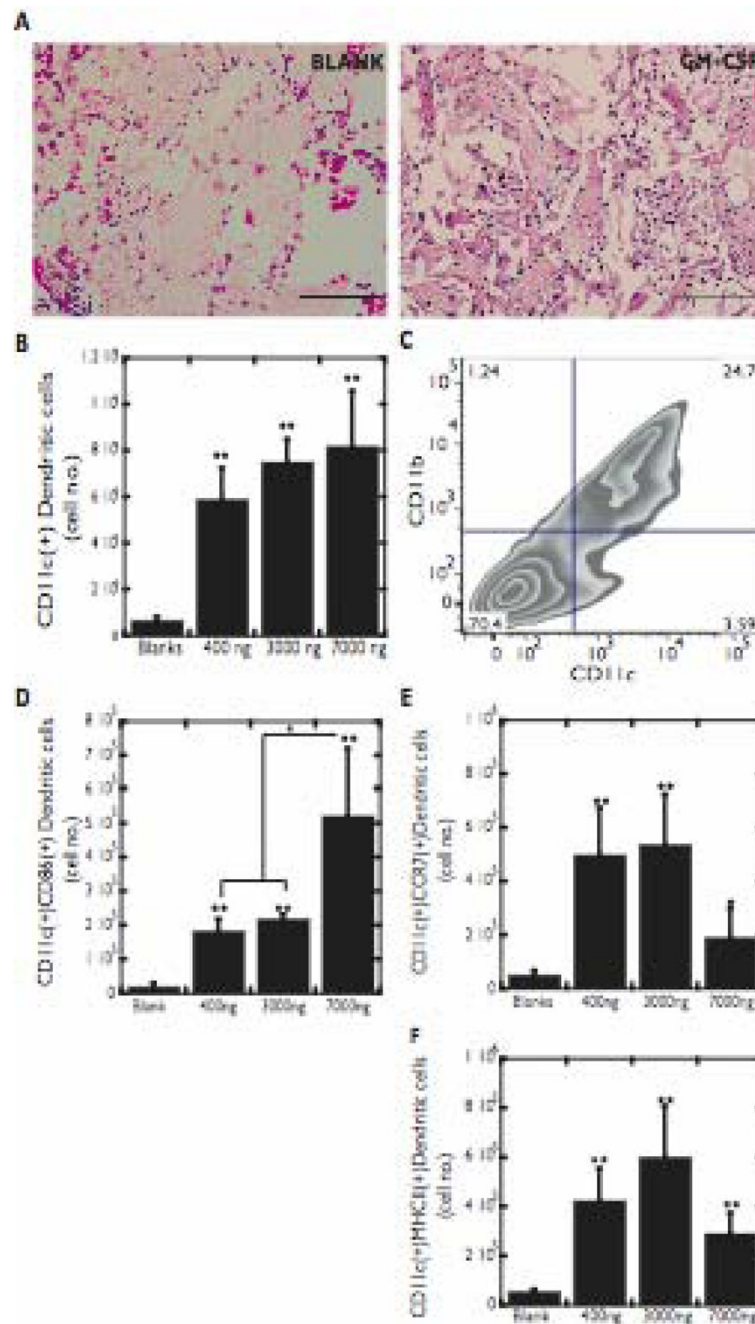


Fig. 1. GM-CSF delivery from PLG matrices promotes CD11b+ DC recruitment and activation
 (A) H&E staining of sectioned PLG scaffolds explanted from subcutaneous pockets in the backs of C57BL/6J mice after 14 days: Blank scaffolds (BLANK), and GM-CSF (3000 ng) loaded scaffolds (GM-CSF). (B) The number of CD11c(+) DCs isolated from PLG scaffolds at day 14 after implantation in response to doses of 0, 1000, 3000 and 7000 ng of GM-CSF. (C) FACS plots of cells isolated from explanted scaffolds and stained for CD11c and CD11b. Cells were isolated from PLG matrices incorporating 3,000ng of GM-CSF at day 10 post-implantation. Numbers in FACS plots indicate the percentage of the cell population positive for CD11c and CD11b or for both markers. (D) The number of CD11c(+)CD86(+), CD11c(+)CCR7(+), and CD11c(+)MHCII(+) DCs isolated from PLG scaffolds at day 14 after

implantation in response to doses of 0, 400, 3000 and 7000 ng of GM-CSF. Values in B and D represent mean and standard deviation (n=4 or 5). * $P < 0.05$ ** $P < 0.01$ as compared to blank matrices unless otherwise noted.

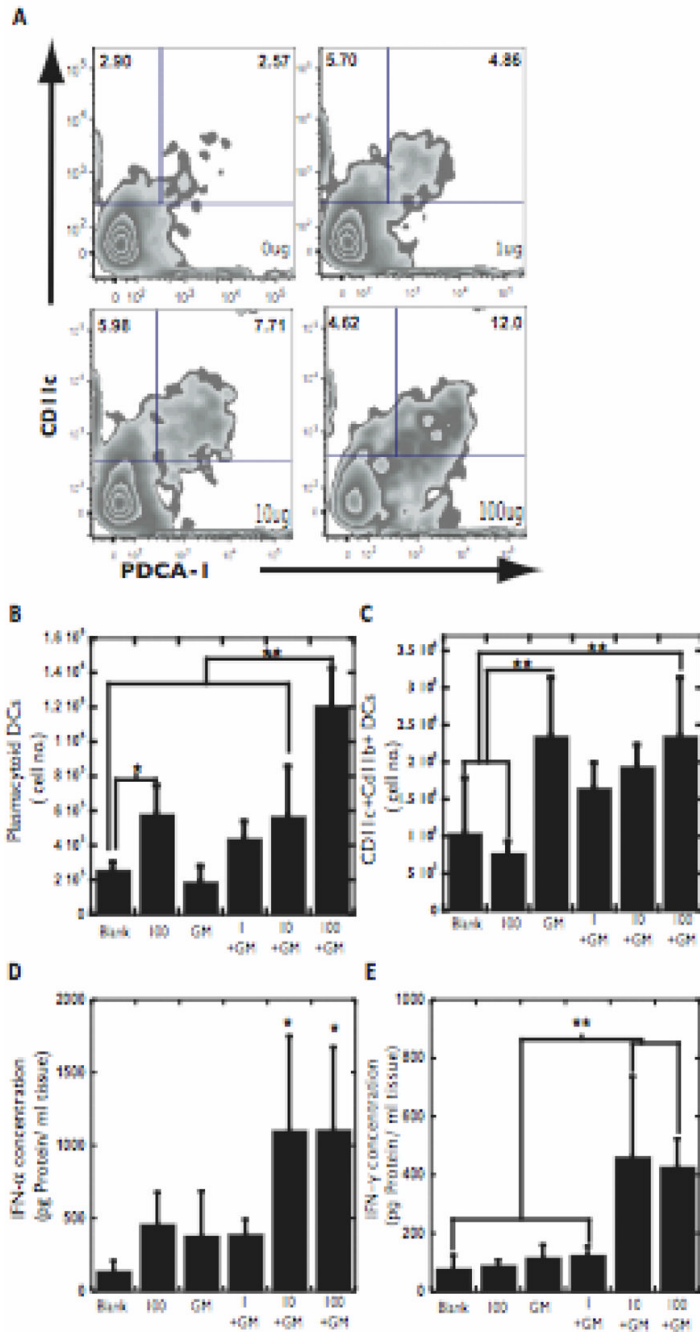


Fig. 2. CpG-ODN and GM-CSF delivery from PLG matrices promotes plasmacytoid DC generation and the production of anti-tumor cytokines

(A) FACS plots of cells isolated from explanted scaffolds and stained for the plasmacytoid DC markers, CD11c and PDCA-1. Cells were isolated from PLG matrices incorporating 0, 1, 10, and 100 µg of CpG-ODN at day 10 post-implantation. Numbers in FACS plots indicate the percentage of the cell population positive for CD11c only or for both markers. The number of (B) plasmacytoid DCs, and (C) CD11c(+)/CD11b(+) cDCs at day 10 post-implantation in blank scaffolds (Blank) or in response to doses of 100 µg (100) of CpG-ODN or 3000ng GM-CSF alone (GM) or GM-CSF in combination with 1 (1+GM), 10 (10+GM), or 100 µg (100+GM) of CpG-ODN. The in vivo concentrations of (D) IFN-α, and (E) IFN-γ at Day 10 post

implantation at the implant site of blank PLG matrices (Blank), or matrices loaded with 3000ng GM-CSF alone (GM) or 10 μ g or 100 μ g (100) of CpG-ODN alone or GM-CSF in combination with 10 (10+GM), or 100 μ g (100+GM) of CpG-ODN. Values in B-E represent mean and standard deviation (n=4 or 5). * $P<0.05$ ** $P<0.01$ as compared to blank matrices unless otherwise noted.

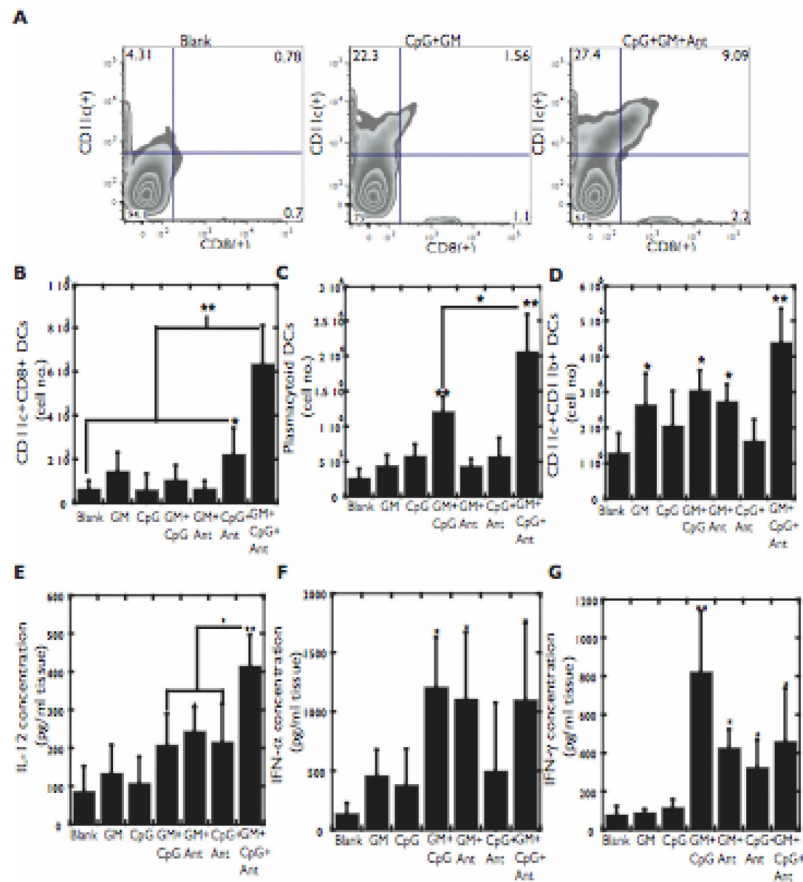


Fig. 3. Tumor lysate, CpG-ODN, and GM-CSF co-delivery from PLG matrices stimulates CD8+ DC generation and IL-12 production

(A) FACS density plots of CD11c and CD8 staining of cells infiltrating Blank PLG matrices (blank) or matrices loaded with 3000ng GM-CSF and 100 μ g CpG-ODN without (CpG+GM) or with tumor lysates (CpG+GM+Ant) at day 10. Numbers in FACS plots indicate the percentage of the cell population positive for CD11c and CD8 or for both markers. (B-D) The number of (B) CD11c(+)CD8(+) cDCs, (C) plasmacytoid DCs, and (D) CD11c(+)CD11b(+) cDCs at day 10 after implantation in blank matrices (Blank) and in response to 3000ng GM-CSF (GM) or 100 μ g CpG-ODN (CpG) alone or in combination (CpG+GM) or co-presented with tumor lysates (GM+Ant, CpG+Ant and CpG+GM+Ant). (E-G) The in vivo concentration of (E) IL-12, (F) IFN- α , and (G) IFN- γ at day 10 after implantation in blank matrices (Blank) and in response to doses of 3000ng GM-CSF (GM) or 100 μ g CpG-ODN (CpG) alone or in combination (CpG+GM) or co-presented with tumor lysates (GM+Ant, CpG+Ant and CpG+GM+Ant). Values in B-G represent mean and SD (n=4 or 5). * $P < 0.05$ ** $P < 0.01$, as compared to blank matrices, unless otherwise noted.

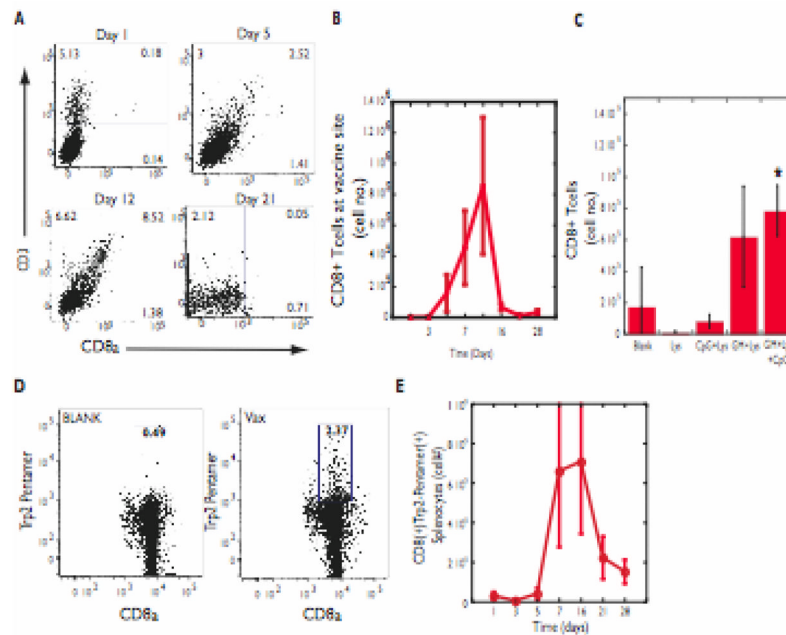


Fig. 4. Tumor lysate, CpG-ODN, and GM-CSF co-delivery in PLG matrices stimulates potent local and systemic CD8+ cytotoxic T cells

(A) FACS plots of cells isolated from explanted matrices and stained for the cytotoxic T cell markers, CD3 and CD8a. Cells were isolated from PLG matrices with 3000ng GM-CSF, 100 μ g CpG-ODN and tumor lysates at days 1, 5, 12 and 21 after implantation. Numbers in FACS plots indicate the percentage of the cell population that was either single positive for CD3 (upper left quadrant of each plot) or CD8 (lower right), or double positive for both markers (upper right). (B) The total number of CD3(+)CD8(+) cytotoxic T cells isolated from PLG matrices loaded with GM-CSF, CpG-ODN and tumor lysates as a function of time after implantation. (C) The number of CD8 T cells at day 12 after implantation in blank scaffolds (Blank) or in response to lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). (D) FACS plots of splenocytes of naïve mice and mice vaccinated with PLG vaccines containing 3000ng GM-CSF, 100 μ gCpG-ODN, and tumor lysates at day 16 post-implantation. Cells were stained with anti-CD8-PE Ab, and Kb/TRP2 pentamers. The gates represent the TRP2-specific, CD8(+) T cells and numbers provide the percentage of gated cells. (E) The total number of TRP2-specific, CD8(+) T cells in the spleens of mice vaccinated with PLG matrices loaded with GM-CSF, CpG-ODN and tumor lysates as a function of time after implantation. Values in B, C and E represent mean and standard deviation (n=4 or 5). * $P < 0.05$ as compared to all other experimental conditions.

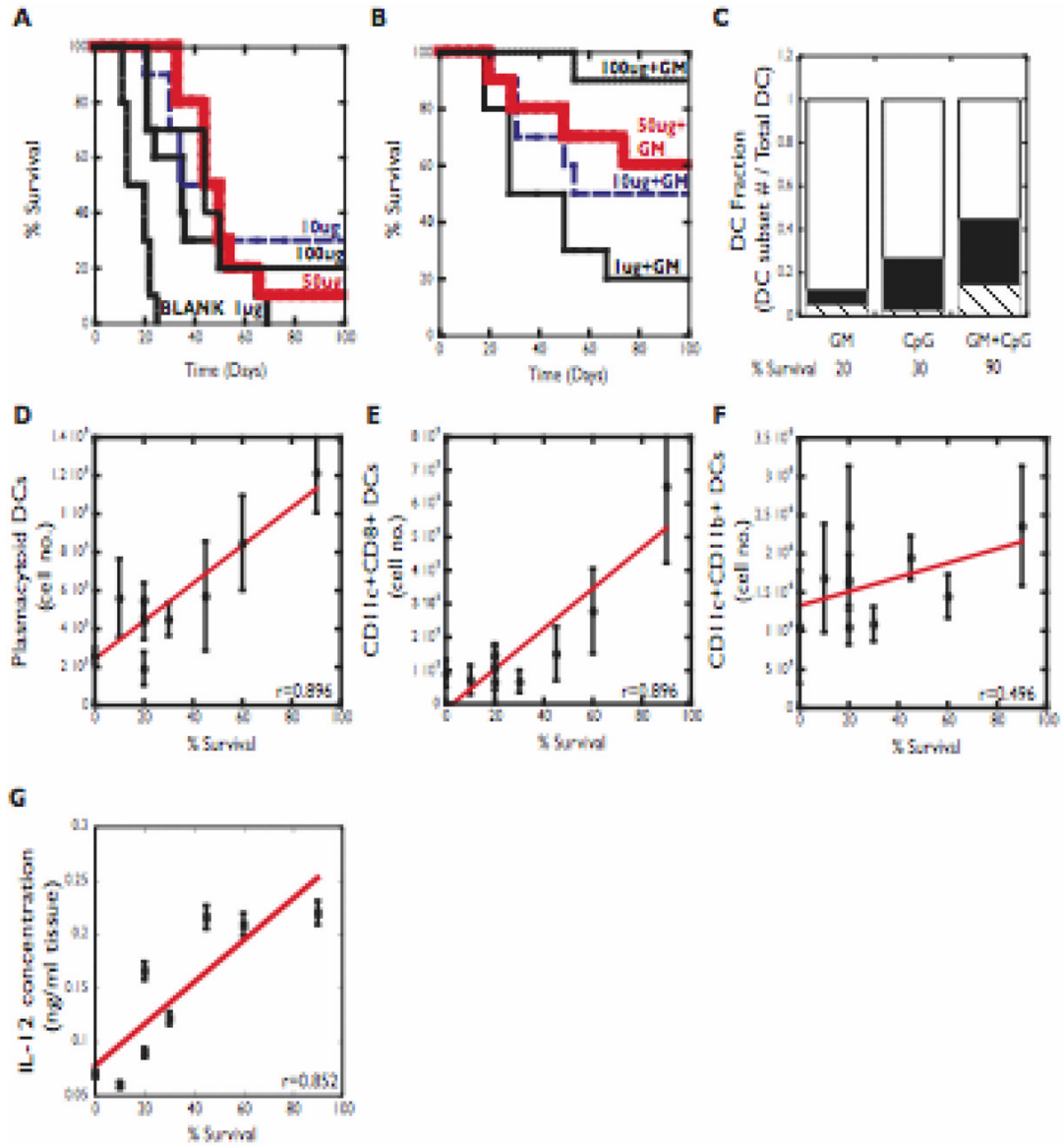


Fig. 5. Tumor protection stimulated by engineered PLG matrices is correlated with DC subsets and IL-12 production

Survival times of mice vaccinated with PLG vaccines 14 days prior to B16-F10 melanoma tumor challenge (10^5 cells). (A) A comparison of survival times in mice treated with blank PLG matrices or with PLG matrices loaded with tumor lysates and 1, 10, 50 or 100 μ g of CpG-ODN. (B) A comparison of survival times in mice vaccinated with PLG matrices loaded with tumor lysates, 3000ng GM-CSF and either 1, 10, 50 or 100 μ g of CpG-ODN. (C) The fraction of the total CD11c(+) DC population consisting of CD11b(+) cDCs (white bar), PDCA-1(+) pDCs (black bar), and CD8(+) cDCs (striped bar) generated at the PLG vaccine site at day 10. Vaccines were loaded with either 3000ng GM-CSF, or 100 μ g of CpG-ODN alone or in combination. Survival percentages recorded at day 100 after tumor challenge. Plots of the numbers of (D) CD11c(+)CD8(+) cDCs (E) CD11c(+)PDCA-1(+) pDCs, and (F) CD11c(+)CD11b(+) cDCs, and (G) the concentration of IL-12 at the PLG vaccine site at day 10 versus the percent of animals surviving B16-F10 melanoma tumor challenge at Day 100 (survival data taken from experimental conditions in A and B). Values in D-G represent mean and SD

(n=4 or 5). r values in D-F represent the linear correlation coefficient between DC numbers or IL-12 concentration and survival percentage.

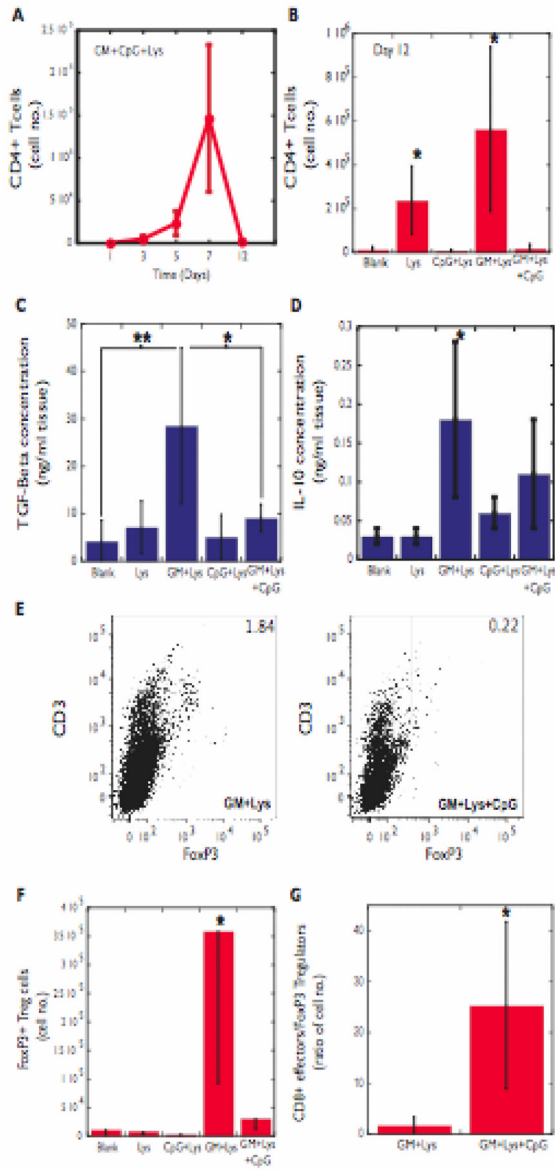


Fig. 6. Engineered PLG matrices attenuate FoxP3+ Tregs and immunosuppressive cytokines
 (A) The total number of CD3(+)/CD4(+) T cells isolated from PLG matrices loaded with GM-CSF, CpG-ODN and tumor lysates as a function of time after implantation. (B) The number of CD4 T cells at day 12 after implantation in blank scaffolds (Blank) or in response to lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). The in vivo concentrations of (C) TGF- β and (D) IL-10 at Day 12 post implantation at the implant site of blank scaffolds (Blank) or scaffolds presenting lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys+CpG). (E) FACS plots of cells isolated from explanted scaffolds and stained for the T regulatory cell markers, CD3 and FoxP3. Cells were isolated from PLG matrices incorporating GM-CSF and lysates without (GM+Lys) or with GM-CSF, lysates and CpG-ODN (GM+Lys+CpG) at day 12 after implantation. Numbers in FACS plots indicate the percentage of the cell population positive for both markers. (F) The number of FoxP3(+) Tregs at day 12 post-implantation in blank scaffolds (Blank) or in response to lysate alone (Lys) or in combination with CpG-ODN (CpG+Lys) or GM-CSF (GM+Lys) or both factors (GM+Lys

+CpG). (G) The ratio of CD8a T cells versus FoxP3(+) Tregs residing within PLG scaffolds loaded with GM-CSF and Lysates (GM+Lys) alone or in combination with CpG-ODN (GM+Lys+CpG) at day 12 post-implantation. Values in A-D, F and G represent mean and SD (n=4 or 5). * $P < 0.05$ ** $P < 0.01$ as compared to all other experimental conditions unless otherwise noted.

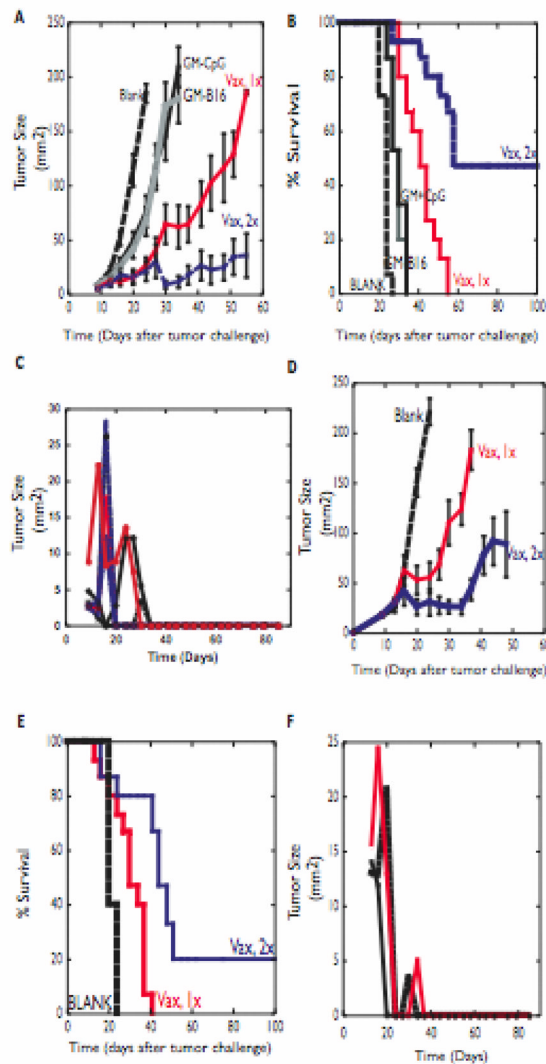


Fig. 7. Engineered PLG matrices stimulate the regression of established melanomas

A comparison of the (A) tumor growth and (B) survival of mice bearing established melanoma tumors (inoculated with 5×10^5 B16-F10 cells and allowed to develop for 9 days) and treated with either blank PLG matrices (Blank), or matrices loaded with 3000ng GM-CSF and 100ug CpG-ODN (GM+CpG). Mice were also treated once (Vax, 1x; at day 9) or twice (Vax, 2x; at days 9 and 19) with PLG matrices incorporating GM-CSF, CpG-ODN and tumor lysates (Vax). Mice were also vaccinated with 5×10^5 irradiated, GM-CSF transduced B16-F10 cells. (C) The individual tumor growth curves for each mouse surviving tumor challenge (5×10^5 cells) after a two-time treatment with PLG vaccines at days 9 and 19. A comparison of the (D) tumor growth and (E) survival of mice bearing established melanoma tumors (inoculated with 5×10^5 B16-F10 cells and allowed to develop for 13 days) and treated with either blank PLG matrices (Blank), or once with PLG vaccines (Vax, 1x; at day 13) or twice (Vax, 2x; at days 13 and 23). (F) The individual tumor growth curves for each mouse surviving tumor challenge (5×10^5 cells) after a two-time treatment with PLG vaccines at days 13 and 23. Values in A & D (A-F; n=15 per condition) represent mean and standard error of the mean.